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Subcellular Compartmentation of the 4-Aminobutyrate Shunt in Protoplasts from Developing Soybean Cotyledons[1]

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The subcellular localization of enzymes involved in the 4-aminobutyrate shunt was investigated in protoplasts prepared from developing soybean [*Glycine max* (L.) Merrill cv Maple Arrow] cotyledons. Protoplast lysate was fractionated by differential and continuous Percoll-gradient centrifugation to separate organelle fractions. Glutamate decarboxylase (EC 4.1.1.15) was found exclusively in the cytosol, whereas 4-aminobutyrate:pyruvate transaminase (EC 2.6.1.19) and succinic semialdehyde dehydrogenase (EC 1.2.1.16) were associated exclusively with the mitochondrial fractions. Mitochondrial fractions also catabolized [U-^{14}C]4-aminobutyrate to labeled succinate.

GABA is widely distributed in all higher plants and represents a significant fraction of the soluble amino acid pool. Although GABA accumulation has been associated with a variety of environmental stress conditions, including hypoxia, temperature shock, and mechanical manipulation (Streeter and Thompson, 1972; Wallace et al., 1984; Satya Narayan and Nair, 1990; Shelp et al., 1995), little is known about the physiological role of GABA and the regulation of its metabolism (Bown and Shelp, 1989). The major route of GABA synthesis is by the direct and irreversible α-decarboxylation of glutamate by GAD (EC 4.1.1.15). Subsequent reversible transamination of GABA with pyruvate (or 2-oxoglutarate) in a reaction catalyzed by GABA-T (EC 2.6.1.19) results in the production of succinic semialdehyde and Ala (or glutamate). The former product can then be oxidized by SSADH (EC 1.2.1.16) to form succinate, an important Krebs cycle intermediate. The conversion of glutamate carbon to succinate by these three reactions is referred to as the GABA shunt.

To date, GAD is the best-characterized enzyme in this pathway. It possesses a calmodulin-binding domain (Baum et al., 1993) and is activated in vitro by calcium-calmodulin (Ling et al., 1994; Snedden et al., 1995). There is considerable evidence suggesting that GAD is exclusively cytosolic (Dixon and Fowden, 1961; Wallace et al., 1984; Satya Narayan and Nair, 1986). In contrast to GAD, less is known about the enzymatic properties of GABA-T and SSADH, and their subcellular locations are controversial (Bown and Shelp, 1989). Previous studies have suggested that GABA-T in callus cells from soybean [*Glycine max* (L.)] cotyledon is mitochondrial (Tokunaga et al., 1976), whereas in pea seedling (Dixon and Fowden, 1961), soybean leaves (Wallace et al., 1984), and potato tuber (Satya Narayan and Nair, 1986), it is both cytosolic and mitochondrial. Satya Narayan and Nair (1986) suggested that both GABA:pyruvate-T and SSADH share a dual mitochondrial and cytosolic location in a 1:3 ratio, respectively. In these studies, unpurified organelle fractions obtained by differential centrifugation of homogenized tissues were used, with no assessment of contamination or organelle breakage. It is possible, therefore, that the appearance of GABA-T and SSADH activities in the soluble/cytosolic fractions was a consequence of mitochondrial breakage during the extraction and fractionation procedures. As a result, the subcellular distribution of enzymes in the GABA shunt, especially GABA-T and SSADH, is both questionable and variable.

Therefore, in this study we tested the hypothesis that enzymes of GABA catabolism are located exclusively in the mitochondrion. By using an improved cellular fractionation technique, we determined the subcellular localizations of the GABA shunt enzymes in organelles derived from developing soybean cotyledonary protoplasts that were purified by continuous Percoll-gradient centrifugation.

MATERIALS AND METHODS

Plant Material

Soybean plants [*Glycine max* (L.) Merrill cv Maple Arrow] were grown in a naturally lighted greenhouse in 9-L pots (five to six plants per pot) containing Pro-mix BX (Les Tourbières Premier Lteé, Rivière du Loup, Quebec, Canada). Natural lighting was supplemented with high-intensity sodium vapor lamps yielding a 16-h photoperiod and a PPFD of 60 μmol m^{-2} s^{-1} at pot level. Each pot was supplied twice weekly with 1 L of one-quarter-strength Hoagland-type nutrient solution (Hoagland and Arnon, 1938), containing 8 mM N, and alternatively with tap water as required.

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Abbreviations: AOA, aminooxyacetate; GABA, 4-aminobutyrate; GABA:pyruvate-T, 4-aminobutyrate:pyruvate transaminase; GABA-T, 4-aminobutyrate transaminase; GAD, glutamate decarboxylase; PLP, pyridoxal 5′-phosphate; SSADH, succinate semialdehyde dehydrogenase.

Fruits were harvested approximately 20 d after anthesis and cotyledons with a length of 8 to 10 mm were collected and used immediately for protoplast isolation.

Protoplast Isolation

The cotyledons were sliced into <1-mm sections and suspended in 20 mL of holding solution consisting of 0.5 M mannitol, 0.1% (w/v) BSA, and 20 mM Mes (pH 6). After 30 min, the holding solution was decanted and replaced with 20 mL of 0.45-μm-filtered digestion solution that contained 1.0% (w/v) cellulase Onozuka R-10, 0.3% (w/v) pectolyase Y-23 (Kanematsu USA Inc., Los Angeles, CA), 0.3% (w/v) drieselase (Sigma), 0.5 M mannitol, 0.1% (w/v) BSA, and 20 mM Mes (pH 5.8). The tissue pieces were incubated in the dark in a nonshaking water bath set at 30°C. After 3 h, the enzyme solution was decanted and replaced with 20 mL of holding solution. Protoplasts released by gentle swirling were sequentially filtered through two nylon meshes (100 and 53 μm) and gently pelleted by centrifugation at 50*g* for 30 min.

Subcellular Fractionation

The protoplast pellet was resuspended in 2 volumes of 0.2 M Suc and passed through a 15-μm pore-size nylon mesh covering the aperture of a 3-mL syringe. The resulting lysate (approximately 12 mL) was initially centrifuged at 50*g* to settle, but not rupture, large starch-containing amyloplasts. After 20 min, the centrifugal force was increased linearly to 2500*g* over 6 min to settle smaller amyloplasts and starch particles. The resultant supernatant (approximately 9 mL), which was enriched in mitochondria and microbodies relative to amyloplasts, was placed onto a 2 to 28% (v/v) linear Percoll (Pharmacia) gradient (26 mL) containing 0.25 M Suc, 0.1% BSA, and 30 mM Mops (pH 7). Centrifugation was at 40,000*g* for 1 h in a Beckman SW28 rotor. Fractions were collected from the top of the gradient using an automated fraction collector.

Enzyme Assays

All enzymes were assayed at 30°C in a final 1-mL volume containing 0.02% (v/v) Triton X-100. Alcohol dehydrogenase (EC 1.1.1.1) was measured according to the method of Kimmerer (1987), isocitrate dehydrogenase (EC 1.1.1.41) according to the method of Bergman et al. (1980), catalase (EC 1.11.1.6) according to the method of Aebi (1984), and Cyt *c* oxidase (EC 1.9.3.1) according to the method of Schnarrenberger et al. (1972). Integrity of the outer mitochondrial membrane was estimated using the Cyt *c* impermeability assay (Neuburger, 1985). GAD (EC 1.4.1.2) activity was determined as the L-[1-^{14}C]glutamate-dependent production of $^{14}CO_2$ essentially as described by Snedden et al. (1992). GABA-T (EC 2.6.1.19) activity was determined as the GABA-dependent production of Ala (or glutamate), with pyruvate (or 2-oxoglutarate) as an amino acceptor (Shelp et al., 1995). The assay mixture contained 100 mM Tris-HCl (pH 8.5), 2 mM GABA, 20 μM PLP, 400 μL of sample, and either 10 mM pyruvate or 10 mM 2-oxoglutarate. Fractions 1 to 7, which represented cytosol, were pooled together and desalted using a Sephadex G-25 column (Pharmacia) equilibrated with 100 mM buffer (Tris or Tricine), 40 μM PLP, and 14 mM 2-mercaptoethanol. This step decreased the production of Ala by glutamate:pyruvate-transaminase (EC 2.6.1.2). The reaction mixture was incubated for 3 h, sulfosalicyclic acid was added to a final concentration of 60 mM, and the precipitate was removed by centrifugation. The supernatant was neutralized with 1 N NaOH and then diluted with HPLC-grade water for amino acid analysis. For each fraction, a control reaction not containing GABA was used. SSADH (EC 1.2.1.16) activity was measured as the difference in NADH production before and after the addition of succinic semialdehyde (Shelp et al., 1995). The final assay mixture contained 100 mM 3-[(1,1-dimethyl-2-hydroxyethyl) amino]-2-hydroxypropanesulfonic acid buffer (pH 9.5), 0.5 mM succinic semialdehyde, 0.5 mM NAD^+, 14 mM 2-mercaptoethanol, and 200 μL of sample.

The density of the Percoll gradient was determined using density marker beads (Sigma) calibrated for 0.25 M Suc. Starch in ethanol-insoluble residues was quantified as described by Taylor et al. (1988). Protein was determined according to the method of Bradford (1976), with BSA as the standard.

Radiolabeling Study

L-[U-^{14}C]GABA (209 Ci mol^{-1}) was obtained from Amersham. The shipping medium was evaporated under a gentle stream of ultrapure N_2 and the amino acid was resuspended in HPLC-grade water. To remove ^{14}C-succinate and other contaminating organic acids from [U-^{14}C]GABA, the preparation was passed through a column containing AG50W-X8 (H^+) resin (Bio-Rad). Organic acids were washed from the column with HPLC-grade water and GABA was eluted with 4 N NH_4OH (Atkins and Canvin, 1971). Subsequently, the washed GABA was dried under a gentle stream of filtered air and resuspended in HPLC-grade water, and the purity was checked by HPLC as described by Shelp et al. (1995).

GABA metabolism by cytosolic (desalted pooled fractions 1 to 7), microbody (fraction 19), and mitochondrial (fraction 25) fractions from the Percoll gradient was determined as the incorporation of ^{14}C radioactivity from L-[U-^{14}C]GABA into succinate. A 1-mL assay mixture contained 100 mM Tricine (pH 8.5), 3 mM malonate, 1 mM [U-^{14}C]GABA (1 μCi), 0.5 mM NAD^+, 80 μM PLP, 0.02% (v/v) Triton X-100, 10 mM pyruvate, and 200 μL of the cytosolic fraction or main microbody and mitochondrial fraction in the presence or absence of 1 mM AOA. The reaction mixture was incubated for 1 h at 30°C in a shaking water bath and the reaction was terminated with 5 mL of boiling 95% ethanol. The samples were subsequently dried under vacuum, resuspended in 2 mL of HPLC-grade water, and partitioned against an equal volume of chloroform. After centrifugation at 2500*g* for 5 min, the aqueous fraction was removed and dried under a gentle stream of filtered air and resuspended in 0.5 mL of HPLC-grade water. Labeled organic acids were analyzed by HPLC according to the method of Tuin and Shelp (1994).

RESULTS

Protoplasts prepared from soybean cotyledons were gently lysed and used as a starting material to separate various organelles. Table I shows the recovery and distribution of protein, selected organelle markers, and GABA-shunt enzymes from lysed protoplasts prior to and following centrifugation at 2500*g*. Eighty-two percent of the starch (amyloplast marker) was recovered in the pellet, whereas 73% of the catalase activity (a microbody marker) and 55% of the Cyt *c* oxidase (a mitochondrial marker) were recovered in the supernatant. Thus, differential centrifugation enriched the supernatant fraction in mitochondria and microbodies relative to amyloplasts. SSADH and GABA:pyruvate-T showed a distribution similar to Cyt *c* oxidase in the pellet and supernatant.

The supernatant fraction obtained from the differential spin was centrifuged through a preformed continuous Percoll gradient to separate the organelles (Fig. 1). No definitive starch peaks were recovered in the gradient, but fractions 24 to 27 near the bottom of the tube contained a small amount of starch (approximately 27 mg; data not shown). Protein was recovered primarily near the top of the gradient, together with 95% of the alcohol dehydrogenase activity (cytosolic marker). Only about 20% of the catalase activity was associated with this cytosolic fraction, indicating minor microbody breakage. The majority of the catalase activity (65%) occurred as a separate broad peak in the middle of the gradient. The remaining catalase activity occurred as a second minor peak at a higher Percoll density (fractions 24–27). This second catalase peak was coincident with peak mitochondrial marker activities (Cyt *c* oxidase and isocitrate dehydrogenase), indicating some microbody contamination of the main mitochondrial fractions. The mitochondria from fractions 24 to 26 had an outer membrane intactness of >90%, as indicated by the Cyt *c* impermeability assay. Negligible mitochondrial marker activity was found in the cytosolic fractions.

The distribution of GAD activity was similar to the cytosolic marker with no activity in the microbody or mitochondrial fractions. In contrast, GABA-T and SSADH had activity profiles very similar to those of the mitochondrial markers, with negative activity (drawn as zero) recovered in the cytosolic fraction. The distribution of GABA:2-oxoglutarate transaminase was identical with GABA:pyruvate-T, but its activity was only 10% (data not shown). Only SSADH showed latency; the activity was stimulated approximately 3-fold by the addition of 0.02% Triton X-100.

To demonstrate further that mitochondria are capable of metabolizing GABA, [U-^{14}C]GABA and unlabeled pyruvate were fed to desalted cytosolic (fractions 1–7) and peak microbody (fraction 19) and mitochondrial (fraction 25) fractions. After 1 h, only the osmotically shocked mitochondrial and microbody fractions catabolized [U-^{14}C]GABA, showing malonate-induced accumulation of labeled succinate at rates of 13.6 and 6.4 nmol h^{-1} fraction^{-1}, respectively (data not shown). Thus, the rate of succinate accumulation by the microbody fraction was only 47% of the mitochondrial fraction. This is in agreement with the extent of mitochondrial contamination of the microbody fraction (43% of the mitochondrial fraction). Suc-

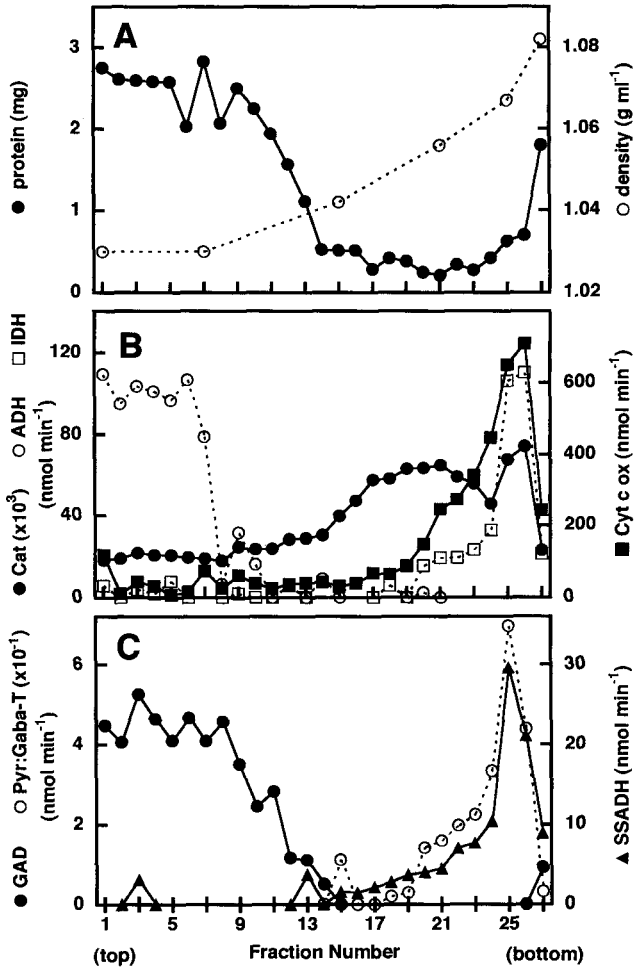

Figure 1. Distribution of some marker and GABA-shunt enzymes among organelle fractions separated by continuous Percoll-density centrifugation of the supernatant fraction from lysed soybean cotyledon protoplasts. ADH, Alcohol dehydrogenase; Cat, catalase; Cyt c ox, Cyt *c* oxidase; IDH, isocitrate dehydrogenase; pyr, pyruvate.

Table I. *Distribution of protein, organelle markers, and GABA-shunt enzymes in lysed protoplasts, and supernatant and pellet fractions resulting from differential centrifugation of lysed protoplasts*

Parameter	Protoplast Lysate	Distribution	
		Pellet	Supernatant
	mg	*mg (% of total recovered)*	
Protein	144	42 (37)	72 (63)
Starch	2512	1078 (82)	241 (18)
	nmol min^{-1}	*nmol min^{-1} (% of total recovered)*	
Catalase (×10^3)	1901	568 (27)	1530 (73)
Cyt *c* oxidase	1492	58 (45)	717 (55)
GAD	124	9.1 (13)	62 (87)
SSADH	232	76 (39)	121 (61)
GABA: pyruvate-T	ND[a]	1.5 (32)	3.3 (68)

[a] ND, Not determined.

cinate formation was not observed in the presence of AOA, an inhibitor of the PLP-dependent GABA-T.

DISCUSSION

Here, a gentle and efficient method was developed to isolate and purify organelle fractions from a protoplast preparation of soybean cotyledons. The preliminary differential centrifugation of the protoplast lysate removed the majority of amyloplasts and enriched the supernatant with mitochondria and microbodies (Table I). Centrifugation of this supernatant through a continuous Percoll gradient separated cytosolic, microbody, and mitochondrial activities (Fig. 1). This technique resulted in very low organelle damage, as indicated by the high yield of intact mitochondria and microbodies.

The distribution of GAD was not associated with any organelle fraction but was found exclusively in the cytosolic fractions (Fig. 1). This result agrees with previous work (Dixon and Fowden, 1961; Wallace et al., 1984; Satya Narayan and Nair, 1986). In contrast, the distributions of GABA:pyruvate-T and SSADH suggest that these activities were located exclusively in the mitochondria and not in the cytosol (Fig. 1). The fact that negative rates were obtained for GABA-T and SSADH in the cytosolic fractions (shown as zero in Fig. 1) perhaps suggests that the substrates for these reactions (i.e. GABA and succinate semialdehyde, respectively) inhibit background reactions. For example, Good and Muench (1992) demonstrated that 1 mM GABA inhibits glutamate:pyruvate transaminase by 24%. Furthermore, accumulation of succinate semialdehyde is known to be toxic to cells (Hearl and Churchich, 1984) and may possibly inhibit other dehydrogenases.

Feeding studies with [U-^{14}C]GABA complemented the findings that GABA-T and SSADH are mitochondrial, because only the mitochondrial fraction (and microbody fraction contaminated with mitochondria), and not the cytosolic fraction, catabolized GABA to succinate in the presence of malonate, an inhibitor of succinate dehydrogenase (Journet et al., 1982). No labeled succinate was detected in the presence of AOA, a potent inhibitor of transaminase reactions (John et al., 1978), demonstrating that GABA catabolism involved GABA-T and SSADH.

Although this work agrees in part with a mitochondrial localization for GABA:2-oxoglutarate transaminase as proposed by Tokunaga et al. (1976), it contradicts the dual cytosolic-mitochondrial distribution suggested for GABA:pyruvate-T (Dixon and Fowden, 1961; Wallace et al., 1984) and the 1:3 ratio for GABA:pyruvate-T and SSADH in the mitochondria and cytosol as suggested by Satya Narayan and Nair (1986). Although the possibility of species- or tissue-dependent isozymes remains, it is likely that the dual localization suggested by previous workers is a direct result of mitochondrial breakage during tissue maceration. According to Douce et al. (1987), maceration is detrimental to mitochondrial integrity, producing envelope-free mitochondria and mitochondria that have resealed following rupture and loss of matrix content.

Whether or not isozymes exist in other plant tissues is unknown; however, it is clear from this study that GABA-T and SSADH in developing soybean cotyledons are confined exclusively to the mitochondria. The latency of SSADH suggests that this enzyme may be membrane bound or complexed with GABA-T. In mammalian systems, both GABA-T and SSADH are confined only to the mitochondria and form a stable enzymatic complex to promote substrate channeling (Hearl and Churchlich, 1984). Therefore, it is probable that this is a recurring motif in biological systems.

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